

Introduction

Conazoles are fungicides used in crop protection and as pharmaceuticals. Conazoles such as triadimefon and propiconazole are hepatotumorigenic in mice, while myclobutanil is not. Previous toxicogenomic studies suggest that alteration of the retinoic acid (RA) metabolism pathway may be a key event in conazole-induced hepatocarcinogenesis. All trans retinoic acid (atRA) (a vitamin A metabolite) is an essential signaling molecule controlling cellular growth, differentiation and apoptosis, and atRA has anti-proliferative and cancer-preventative properties.

In this research, we have used quantitative real-time polymerase chain reaction (qRT-PCR) to examine the expression of specific genes associated with the biosynthesis and metabolism of atRA in the livers from male CD-1 mice. The mice were exposed to triadimefon (1800 ppm; equivalent consumed dose=257 mg/kg/d), propiconazole (2500 ppm; equivalent consumed dose=150 mg/kg/d) or myclobutanil (2000 ppm; equivalent consumed dose=270 mg/kg/d) in the feed for 4, 30 or 90 days. The genes examined were RA biosynthesis genes, *Bcmo1* (β-carotene 15, 15'-monooxygenase), *Aox1* (aldehyde oxidase), *Rdh10* (retinol dehydrogenase) and *Alah1a7* (aldehyde dehydrogenase); the RA catabolism gene, *Cyp26a1* (retinoic acid hydrolase); and the retinol transport and storage gene, *Lrat* (lecithin-retinol acyltransferase). Western immunoblot analysis was used to assess protein expression of *Cyp26a1* in liver microsomes from triadimefon treated mice in the feed study.

To investigate the activity of RA metabolism in livers, *in vitro* metabolism of atRA was performed in liver microsomes from male CD-1 mice following intraperitoneal (IP) injection of triadimefon (257 mg/kg/d) or myclobutanil (270 mg/kg/d) for 4 days. The total RA metabolism activity and *Cyp26a1* protein expression were quantified in liver microsomes from conazole treated mice.

Research Objectives

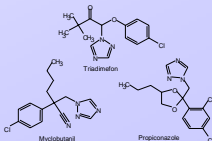
1. Validate transcriptional expression of RA-associated genes in mouse livers from the feed study.
2. Investigate correlation of gene expression between atRA biosynthetic and metabolism genes across time in mouse livers from the feed study.
3. Compare transcriptional and protein expression of *Cyp26a1* gene in livers from triadimefon-treated mice in the feed study.
4. Compare microsomal activity of RA metabolism and *Cyp26a1* protein expression in mouse livers from the IP injection study.

Research Goal

Identify molecular biomarkers associated with RA pathways to elucidate the modes of action of hepatocarcinogenic and non-hepatocarcinogenic conazoles.

Conazoles

- Triadimefon (Bayleton)
 - mouse: hepatocellular tumor
 - rat: thyroid follicular cell tumor
- Propiconazole (Tih)
 - mouse: hepatocellular tumor
 - rat: no thyroid tumors
- Myclobutanil (Eagle)
 - mouse: no liver tumors
 - rat: no thyroid tumors



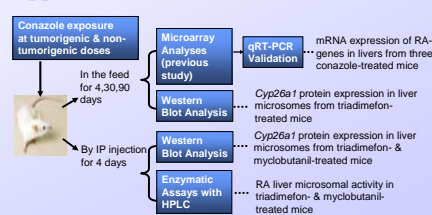
Methods

qRT-PCR Total RNA was isolated from livers using TRI reagent (Molecular Research Center), and then converted to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen). qRT-PCR was performed in a 10 µl reaction (containing 12.5 ng cDNA, TaqMan primer/probe mix and Universal PCR Master Mix) on the ABI Prism 7900 sequence detection system (Applied Biosystems). The fold change of the target gene was calculated using ΔMCT Method (against endogenous control, GAPDH, and the untreated control).

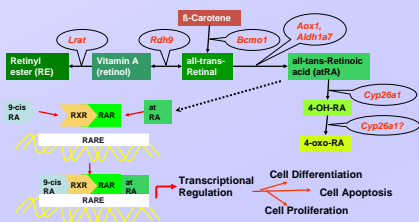
Western Blot Analysis 10 or 20 µg of microsomal protein was fractionated on 4-12% Bis-Tris gels using SDS-PAGE Running System (Invitrogen), and then transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in Tris-buffered saline, and then incubated overnight at 4 °C with primary antibodies of anti-human *Cyp26a1* (Alpha Diagnostic Inc) or anti-Calnexin (BD Bioscience, loading control). The protein expression was revealed by chemiluminescence with ECL Plus Detection System (Amersham Bioscience) and captured by PhorChem Imaging System (Alpha Innotech) following incubations of secondary antibodies (conjugated with HRP) and washing procedures between and after each antibody incubation.

Enzymatic Assays of atRA-Metabolism 1 mg/ml microsomes were pre-incubated at 37 °C in phosphate buffer saline (0.1 M, pH 7.4, final volume of incubation = 500 µl) with 1 mM NADPH for 5 min. The reaction was initiated by addition of 100 nM atRA, and terminated after 10 min with 2 ml mixture of diethyl ether/hexyl acetate. After centrifugation, supernatant fluids were dried under N₂(g), and then dissolved in 500 µl acetonitrile for HPLC analysis.

Approach

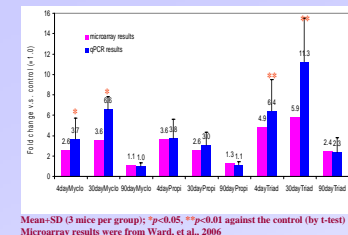


RA-associated Pathways

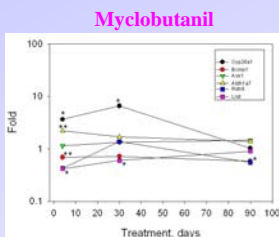
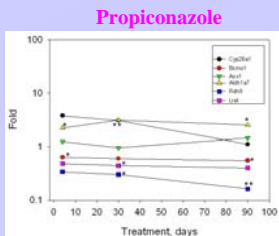
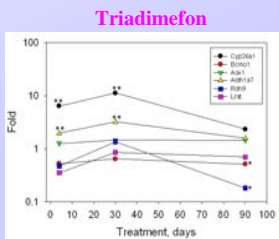


Results

Gene Expression Validation (*Cyp26a1*)

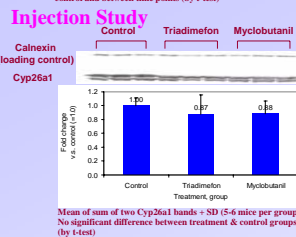
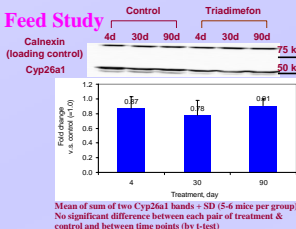


Transcriptional Expression (qRT-PCR)

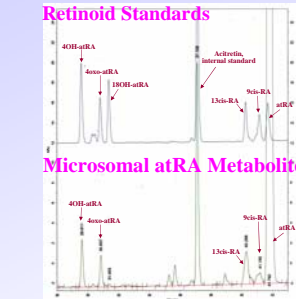


Each value represents mean of 3 mice per group;
*p<0.05, **p<0.01 against the control (by t-test)

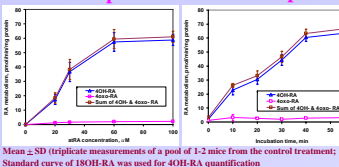
Protein Expression (*Cyp26a1*)



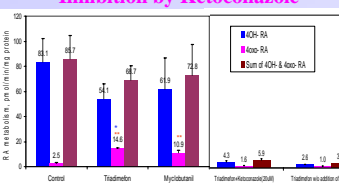
HPLC Chromatograms



RA Metabolism Activity in Liver Dose Response Time Response



RA Metabolism by Microsomes & Inhibition by Ketoconazole



Conclusions

- qRT-PCR results of *Cyp26a1* expression were in a good agreement with our previous microarray genomic findings.
- Overall, transcriptional activities of atRA biosynthetic genes (*Bcmo1* and *Rdh10*), and *Lrat* were correlated with temporal expression of atRA metabolism gene, *Cyp26a1*, for tumorigenic conazoles.
- Triadimefon induced the highest mRNA expression of *Cyp26a1* in livers at selected time points among three conazoles, but protein expression of *Cyp26a1* was not increased during treatments.
- Microsomes from triadimefon- and myclobutanil-treated mice possessed higher atRA catabolizing activities on formation of the 4-oxo-metabolite following a 4 day exposure, while total RA metabolism activities and *Cyp26a1* protein expression were not significantly altered by either conazole, implying other conazole-induced cytochrome P450 enzyme(s) may participate in 4-oxo-RA formation.

Acknowledgements

We thank Dr. Douglas Wolf for his review and sponsorship, and Rachel Grindstaff for her assistance on method development of Western blot analysis.

Reference

Ward, W.O., Delker, D.A., Hester, S.D., Thai, S.-F., Wolf, D.C., Allen, J.W., and Nesnow, S. Toxicol Pathol, 34(7), 863-878, 2006.